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Ecology

Chemical basis of courtship in a beetle (*Neopyrochroa flabellata*): Cantharidin as precopulatory "enticing" agent*

(sexual selection/nuptial gift/pheromone/Spanish fly/Coleoptera: Pyrochroidae)

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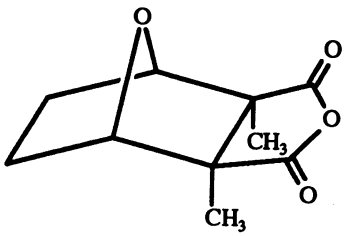
ABSTRACT **Male *Neopyrochroa flabellata* have a natural affinity for cantharidin (Spanish fly). They are attracted to cantharidin baits in the field and feed on the compound if it is offered to them in the laboratory. Males that ingest cantharidin secrete cantharidin from a cephalic gland. Females sample secretion from this gland during courtship and mate preferentially with males that had fed on cantharidin. Cantharidin-unfed males can be rendered acceptable to females if cantharidin is added to their cephalic gland.**

We here describe the behavior of a beetle, *Neopyrochroa flabellata* (family Pyrochroidae), that protects its eggs by use of a noxious chemical. The substance, cantharidin (Spanish fly), initially procured by the male from an exogenous source, is transmitted by the male to the female at mating and by the female to the eggs. The male obtains the chemical by ingestion, and as proof of having acquired it, presents a sample of the substance to the female at courtship in the form of a secretory "offering." Thus "enticed," the female mates with the male and in the process receives, as part of the sperm package, the bulk of the male's cantharidin supply.

In this, the first of two papers on this behavior, we deal with the role of cantharidin as a precopulatory offering. In the companion paper (1) we document the copulatory transmission of cantharidin and its transfer to the eggs. Preliminary findings pertaining to this study have been published (2).



cantharidin

We discovered the affinity of *N. flabellata* for cantharidin when we noted adult males to be attracted to outdoor traps baited with the chemical (3). The experiments we present here were undertaken in the laboratory with adults of both sexes. Specifically, we demonstrate that (*i*) males avidly consume cantharidin; (*ii*) following cantharidin ingestion, males produce a cantharidin-rich secretion from a cephalic gland, which the female samples during courtship; (*iii*) males able to provide such cantharidin offerings are accepted by females; and (*iv*) males devoid of cantharidin tend to be rejected by females, but can be rendered acceptable if cantharidin is added to their cephalic gland.

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MATERIALS AND METHODS

Beetles. Most beetles were reared from larvae and pupae, collected in Sauk County, WI, in their typical larval habitat: the underside of partially buried logs, especially *Quercus* spp., beneath bark and within decaying woody material. Larvae were brought to the laboratory and reared to the pupal stage either on their natural woody substrate or on an artificial diet (4). Pupae and larvae were confined singly in small dishes until adult emergence.

Some males were taken as adults in Sauk County, WI, in baited Lindgren traps (5) hung from trees in wooded areas. The baits (≈2 mg cantharidin on discs of filter paper placed in the center of screened enclosures) were hung directly beside the traps. Attracted males fell into the traps without chance of contacting the bait.

A few adult males were caught near Ithaca, NY, at ultraviolet light sources to which they were attracted at night.

Adult beetles of both sexes were maintained individually in small dishes on a diet of water and dilute honey solution, both presented on cotton wads. Adult survival was 4–5 weeks. Experiments and chemical analyses were performed at our Cornell laboratories.

Chemical Analyses. Quantification of cantharidin was effected by a described procedure (6). In accord with this procedure, each sample was subjected to a preliminary digestion with 12 M hydrochloric acid; the cantharidin was recovered by continuous extraction with dichloromethane. The dried extract was concentrated to a small volume by using a stream of nitrogen. Benzophenone was added to each sample as an internal standard. Gas chromatographic analyses of standard solutions of cantharidin and benzophenone showed a linear flame ionization detector response over the range of 0.1 to 2.0 μg, although it is clear (6) that the linear range is actually much greater.

Statistics. Statistical analyses were performed using SYSTAT (7). All *t* tests compared means of independent samples. Following ANOVAs that indicated significant differences ($P < 0.05$), multiple comparison tests were performed (Tukey–Kramer, experiment-wide $\alpha = 0.05$). Values (including those in the figures) are presented as mean ± SEM.

Cantharidin Feeding. Preliminary testing had shown males to feed avidly on cantharidin. When a few micrograms of the chemical were introduced into a male's dish, the beetle turned promptly toward the offering, and upon contacting the crystals proceeded immediately to ingest them.

Experimental males designated as cantharidin-fed ($n = 34$) were offered crystalline cantharidin on small glass coverslips (18 mm square) (Fig. 1*A*). The chemical was applied to the coverslips in measured volumes of dichloromethane solution

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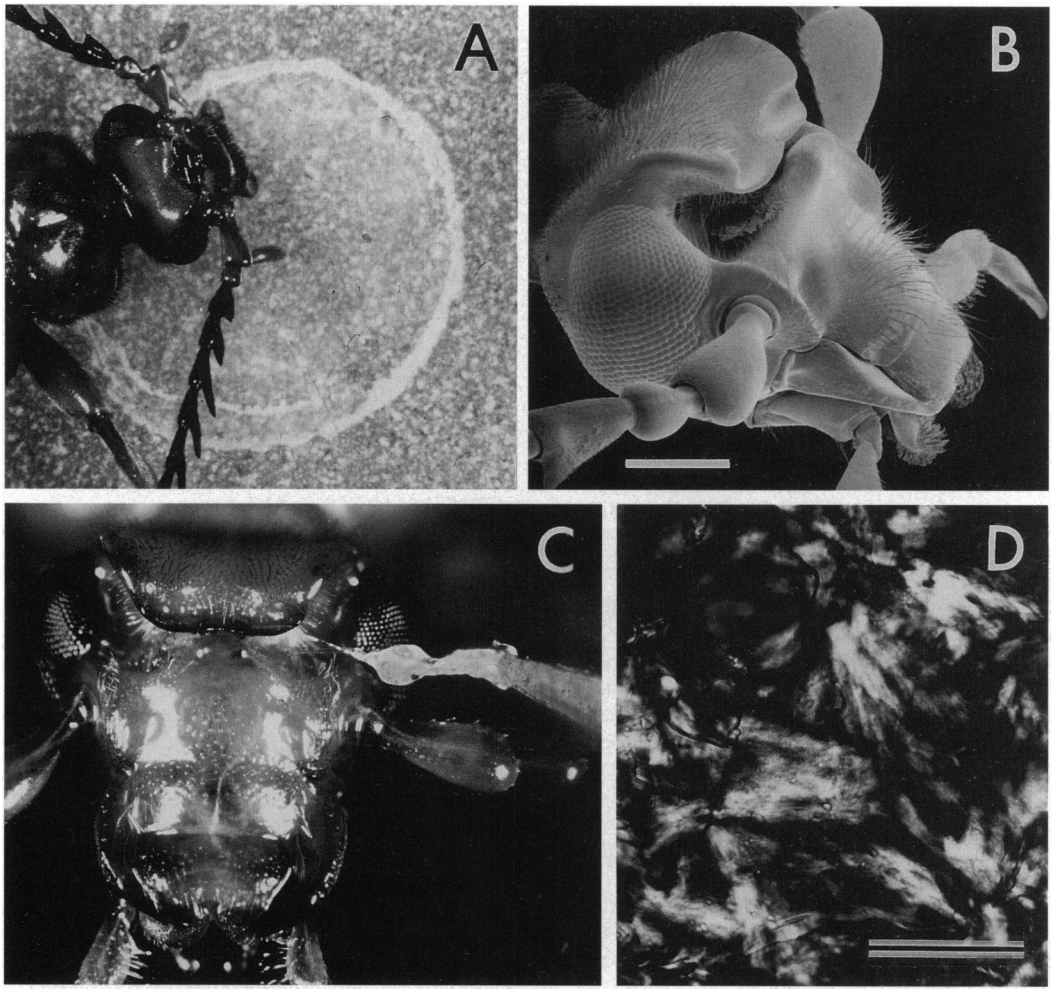

FIG. 1. Male *N. flabellata*. (*A*) Feeding on crystaline cantharidin. (*B*) Head, showing glandular cleft on frons. (*C*) Secretion being extracted from glandular cleft with a fine pin. (*D*) Cantharidin crystals in secretion from a cantharidin-fed male (photographed in transmitted polarized light). [Bars = 0.5 mm (*B*) and 0.01 mm (*D*).]

and was presented to the beetles after evaporation of the solvent. Total cantharidin offered to individuals ranged from 5 to 1350 μg and was not necessarily provided all at once, but as a series of offerings over a span of 1–14 days. Mean dosage per beetle was 521 ± 58 μg, given over a period of 5.7 ± 0.6 days. The first offering was usually given within at most a few days after a male's emergence from the pupa, or of being field-caught. The reason for varying the amount of cantharidin offered is that we had no way of predicting prior to initiation of the study how much of the substance male *N. flabellata* would ingest.

Initial cantharidin offerings of 5 or 50 μg were usually consumed uninterruptedly by the beetles until no visible crystals remained. Larger first offerings, or later offerings presented as part of a series, were sometimes not completely consumed. Total quantities of cantharidin actually ingested by individual males were therefore likely to have been lower in some cases than the sum of amounts offered.

Experimental males designated as cantharidin-unfed (n = 41) were given no access to cantharidin.

The Cephalic Gland. This gland, present in the head of the male only, consists of a deep transverse frontal cleft, medially narrow and pronouncedly flared at each end (Fig. 1*B*). Pilose within, the cleft ordinarily contains a sticky translucent secretion that is readily extractable by probing with a fine needle (Fig. 1*C*). Samples of secretion collected for cantharidin analysis were obtained by such procedure.

Cantharidin Addition to the Cephalic Gland. Individual cantharidin-unfed males were artificially subsidized with cantharidin by picking up a few crystals of the substance with the tip of a needle and inserting the needle briefly into the cephalic glandular cleft. The crystals readily adhered to the secretion in the cleft. The amount of cantharidin added to the beetles in this fashion was estimated, by actual weighings (n = 4) of needle-loads of the chemical, to have been 3.9 ± 1.4 μg. Males thus subsidized with cantharidin were used in courtship trials within the day of treatment.

Courtship Trials. These were staged in Petri dishes (90 mm diameter, 20 mm height), the inner bottom surface of which was sandblasted to provide the beetles with an appropriately roughened locomotory substrate. A trial consisted of introducing first a male into the dish (a reared virgin or a field-collected individual that had been kept unmated) and then, within seconds, a virgin female. Trials lasted 5 min or until a mating pair uncoupled and were videotaped for subsequent behavioral analysis. Specific events in the courtship interaction were identified from the tapes, and their sequence and frequency of occurrence were noted.

Trials were done with males of three types: cantharidin-fed (n = 21), cantharidin-unfed (n = 24), and treated by addition of cantharidin to the gland (n = 14). None of the beetles was used in more than one trial.

For each category of trial, data were summarized in the form of a behavioral chart, in which numbers (and arrow widths) give the frequency of occurrence of the various behavioral

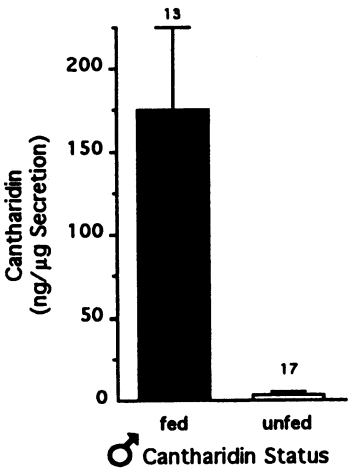

FIG. 2. Concentration of cantharidin in cephalic gland secretion of cantharidin-fed and cantharidin-unfed male *N. flabellata*. Numbers above columns are sample sizes.

sequences (that is, the sum total of times that the sequences occurred in the trials of that category).

For selected events, a calculation was made of the proportion of times that that event led directly to a particular behavioral sequel as opposed to alternative sequels. To this end, the number of times the event led to the designated sequel was divided by the frequency of occurrence of the event. The calculation was repeated for each trial of a category, providing a basis for calculation of the mean. Two sequences were analyzed in this fashion: encounter to breakup, and gland-presentation to gland-sampling.

Duration of events (copulation, gland sampling) was determined directly from the time marker on the videotapes. Where gland sampling occurred twice in a trial ($n = 5$), the recorded value was the mean of the two durations.

RESULTS

Cephalic Gland Secretion. Weighed samples of secretion were obtained from cantharidin-fed males (sample mass = 9.2 $\pm$ 1.9 μg, $n = 13$) and cantharidin-unfed males (sample mass = 8.8 $\pm$ 1.2 μg, $n = 17$), and the samples were analyzed for cantharidin content.

While the latter samples contained virtually no cantharidin, the former contained on average $\approx$17 percent cantharidin (Fig. 2). That concentration is higher than one might expect to be soluble in the secretion, and indeed, microscopic examination of cantharidin-laden secretion samples revealed a dense packing of crystals (Fig. 1*D*), such as were entirely absent from the secretion of cantharidin-unfed controls.

If the $\approx$9 μg samples that we obtained for analysis are a reflection of the approximate amount of secretion in the gland, then the glandular cantharidin load of a cantharidin-fed beetle can be expected to be in the order of 1.5 μg.

Further analysis of the data revealed that cantharidin translocation to the gland, following feeding, must occur relatively quickly. The one cantharidin-fed individual that received a single offering of cantharidin (100 μg) and was "milked" of secretion the next day had cantharidin in its secretion at a concentration of 135 ng/μg. A single feeding on cantharidin can therefore suffice to bring the cantharidin content of a gland to near its mean value.

Courtship Trials. The results with the three categories of male are shown in Fig. 3.

Cantharidin-fed males. Courtship with these males (Fig. 3*A*) led to copulation in 20 of 21 trials. The events before mating proceeded with little variation, in the sequence denoted by the widest arrows. Following the initial encounter of the pair, which involved typically the male moving toward the female rather than *vice versa*, the male oriented itself face-to-face with respect to the female, as if to present its gland (Fig. 4*A*). There typically followed a brief pause, upon which male and female abruptly raised their front end, the male placed his fore- and mid-legs upon the female's flanks, and the female grasped the male's head by inserting her mandibles into the flared ends of the male's glandular cleft. The pair remained locked in this fashion (Fig. 4*B*) for a protracted period, during which the female's mouth parts underwent motions suggestive of feeding. Following such "sampling" of the gland, the female relinquished her hold on the male, upon which the male quickly mounted the female and attempted to effect intromission. The female typically remained passive during these attempts, which usually met with success within seconds (Fig. 4*C*). For a lengthy period the pair remained quiescent, with the male astride the female. Prior to end of copulation, the male dismounted and became flaccid, whereupon the female commenced walking and the pair became uncoupled.

Cantharidin-unfed males. With these males (Fig. 3*B*) only 3 of 24 trials led to copulation. There was a high incidence of male–female encounters, but also a high incidence of breakup. Breakups were often abrupt, after no more than brief antennal contact. Mountings occurred mostly as a sequel to encounters,

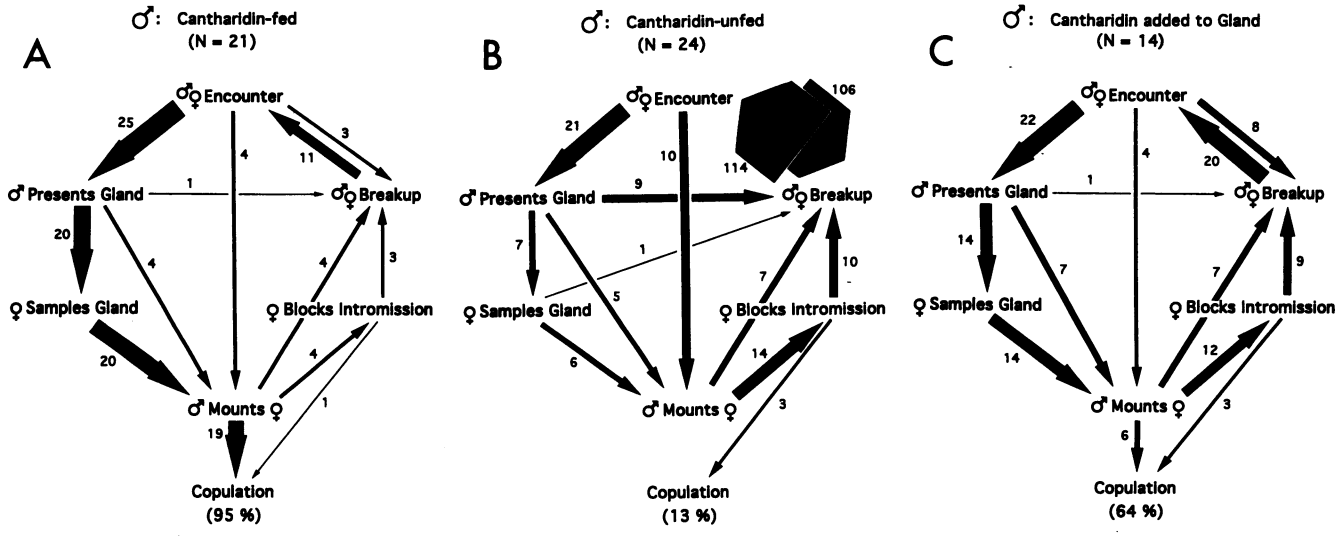

FIG. 3. Flow of events in courtship trials with the three categories of *N. flabellata* males.

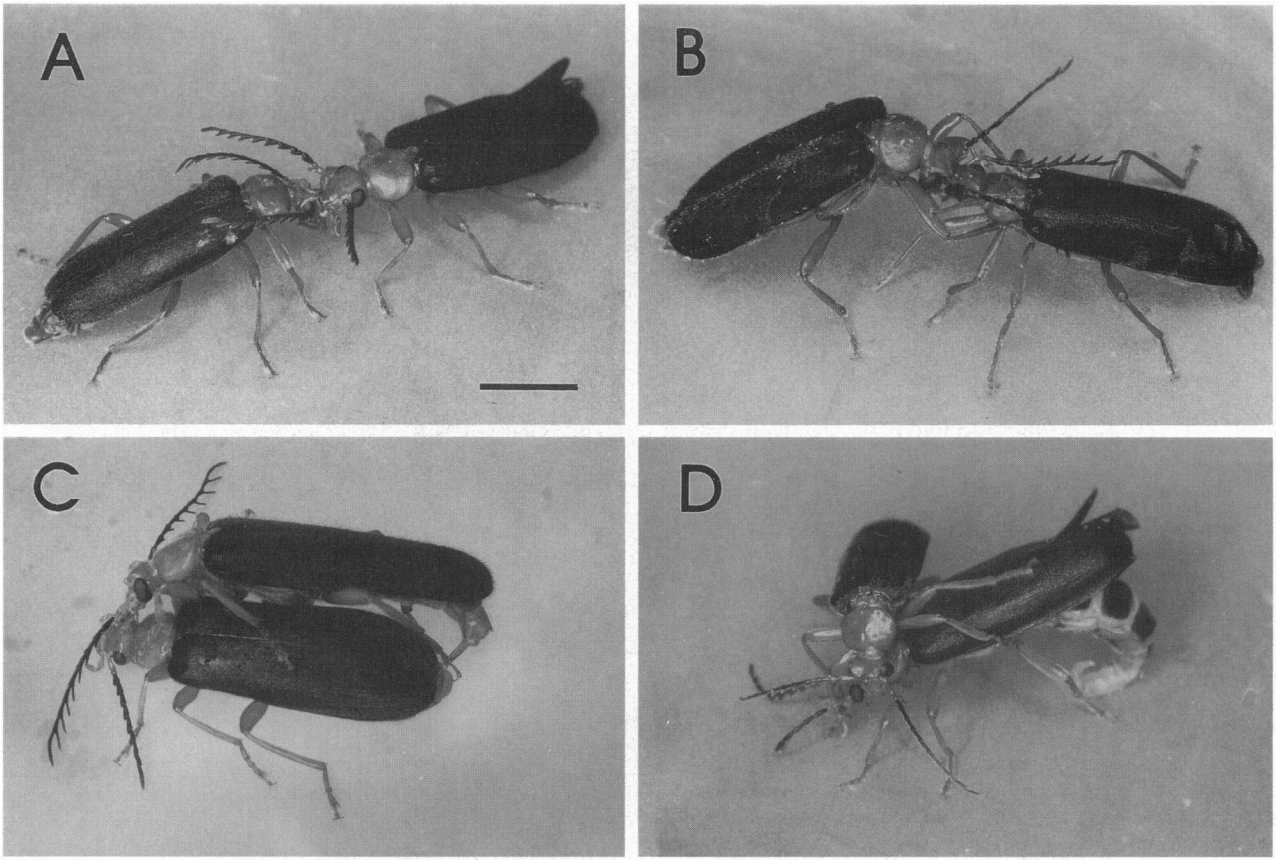

FIG. 4. Events in *N. flabellata* courtship. (*A*) Male (left) presents gland. (*B*) Female (right) samples gland. (*C*) Male mounts female (copulation). (*D*) Female coiling her abdomen and assuming the "blocks intromission" stance in response to attempted mounting by a male. (Bar = 0.5 cm.)

without being preceded by gland samplings, and did not in a single case lead directly to copulation. Gland samplings were, in fact, rare. In a relatively high incidence of cases, the female, when mounted, attempted to block male intromission. She did this by coiling her abdomen forward (Fig. 4*D*), in such way as to render her genitalia inaccessible to those of the male. The three matings that did occur were each preceded by such thwarting attempts on the part of the female.

Males with cantharidin added to the gland. The results with these males (Fig. 3*C*) were similar to those with cantharidin-fed males. Incidence of copulation was high (9 of 14 cases), as was incidence of gland sampling. Gland sampling frequently led to mounting, and mounting relatively often led directly to copulation. Breakup following encounters was relatively rare.

Parameter comparisons. Fig. 5 gives mean values for some of the courtship data, and statistical comparisons. As is clear (Fig. 5*A*), male–female encounters were most likely to lead to breakup if males were cantharidin-unfed. Such males were also less likely to have their glands sampled following gland presentation than cantharidin-fed males. Males that had cantharidin added to their glands were as likely to be sampled after gland presentation as males that were cantharidin-fed.

Duration of gland sampling (Fig. 5*B*) was markedly brief with cantharidin-unfed males. Microscopic examination of the glandular clefts of several such males (individuals that had been noted to be sampled) after the trials, revealed their glands still to contain secretion (as evidenced by scraping with a needle). Males of the other two categories (cantharidin-fed and cantharidin added to gland) similarly examined after trials, proved to have no detectable secretion in their cleft.

Duration of copulation was not significantly different with the three types of males: cantharidin-fed (6.8 ± 0.4 min, n = 20), cantharidin-unfed (5.0 ± 0.9 min, n = 3), and cantharidin added to gland (5.6 ± 0.5 min, n = 9) (ANOVA, P = 0.13).

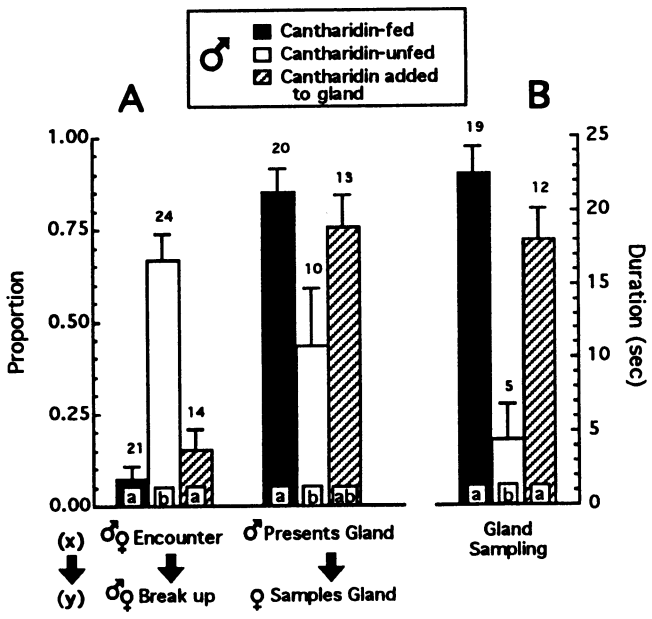

FIG. 5. (*A*) Proportion of times that event **x** led to event **y**, rather than to alternative events, in courtship trials with the three types of male. For both sequences, ANOVA (using arcsine-square root transformed proportions) detected differences among means ($P < 0.02$, in both cases). (*B*) Duration of gland sampling (Fig. 4*B*) in courtship trials with the three types of male. ANOVA detected differences among means ($P < 0.001$). For both *A* and *B*, columns not sharing letters are significantly different (experiment-wide $\alpha = 0.05$); numbers above columns are sample sizes.

DISCUSSION

Feeding on cantharidin evidently leads to translocation of the chemical to the cephalic gland in male *N. flabellata*, and glandular possession of cantharidin gives the male a substantially enhanced chance of being accepted in courtship. The small fraction of ingested cantharidin that finds its way into a male's gland, amounting to at most a few micrograms, suffices to "entice" the female. The female ingests the cantharidin-containing secretion, but she appears able to detect whether or not a male is cantharidin-laden, and therefore acceptable or unacceptable, before making oral contact with the glandular product. Indeed, rejection of cantharidin-unfed males occurred frequently as a sequel to encounters, or of gland presentations, prior to actual sampling of the secretion. The fact that males in which cantharidin was added to the glands tended to be acceptable indicates that mere glandular possession of the chemical, without additional systemic possession, suffices for males to be diagnosed as desirable. Cantharidin, in the context of close-range courtship interaction in *N. flabellata*, therefore appears to function as a conventional, olfactorily-mediated, excitatory pheromone. What is unconventional is that the males derive the chemical from an exogenous source and that the compound serves also, to the extent that it is transmitted to the female and eggs, for defense (1).

We find it ironic that cantharidin, the notorious Spanish fly of folklore, a compound long misused as a purported aphrodisiac by humans (8), should actually function in an aphrodisiac-like capacity in an insect.

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